REMARKS UNDER 37 CFR §1.116

Applicants acknowledge the current status of the claims as reported in the Office Action mailed 12 December 2002. Claims 4-12 and 14-61, are pending; claims 1-3 and 13 are cancelled; and claims 39-43, and 47-60 are withdrawn from consideration

Applicants acknowledge the requirement for a new title is held in abeyance.

Applicants note the Examiner's statement concerning Applicants' claim for domestic priority under 35 USC §119(e) to provisional application Ser. No. 60/181,608. Applicants acknowledge the Examiner's statement that Applicants' priority document provides proper support under 35 USC §112 of the IL-18 epitope defined by SEQ ID NO:1 and human monoclonal antibodies to this IL-18 epitope or to intact IL-18. For the record, Applicants disagree with the Examiner's characterization of priority to other specific embodiments of Applicants' invention, however. Response to these statements are made below where appropriate.

Applicants acknowledge Examiner's removal of rejection of claims 1-3, 11-15 and 46 under 35 USC §112, second paragraph, claims 11-12, 14-15,and 16-21 under 35 USC 102(a), and claims 11-12 and 14-15 under 35 USC 102(b).

Claims 39-43, and 47-60 are hereby canceled without prejudice. Applicants reserve the right to prosecute canceled subject matter in a later-filed continuation application, which properly claims the benefit of this application.

Claim 22 is amended, deleting the term 'CDR domain' from the phrase "variable region CDR domain". This amendment is made to avoid any latent ambiguity regarding claim recitation of what Applicants claim as their invention. This amendment is supported throughout the specification as filed, and particularly at page 11, lines 27-38 and page 12 lines 1-30. No new matter is added.

Attached hereto as **Appendix A** is a marked-up version of the changes made to the claims by amendment under 37 CFR § 1.121(c)(1)(i). Also attached as **Exhibit A** and **Exhibit B** are references provided in support of Applicants' response remarks presented below.

Reconsideration and allowance of the pending claims in light of the foregoing amendments and the following remarks are respectfully requested.

Claim rejections under 35 USC §112 first paragraph

In the Office Action, at page 3-4, paragraph 8, claims 22-38 are rejected under 35 USC §112 first paragraph, as containing subject matter not described in such a way as to convey to one skilled in the art that the Applicants were in possession of the claimed invention at the time the application was filed.

Specifically the Examiner asserts, that the ordinary artisan would not recognize Applicants to be in possession of an antibody or an antigen-binding fragment that binds IL-18 or an epitope of IL-18 unless all six CDRs are defined. Applicants respectfully disagree.

It is well established that "The specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v.American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984)." [MPEP § 2164.05(a), see also § 2164.01 and § 2182].

In the specification as filed, on page 12, lines 4-28, Applicants teach and disclose examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR).

Applicants rely upon the scientific fact that not all CDRs of an antigen binding site may be utilized, or even necessary, in binding a specific antigen, and that functional antibody fragments comprising fewer than six CDRs is well known by practitioners skilled in the art. In addition to the disclosure of Ward *et al.*, (1989) *Nature* 341:544-546 (previously cited by Applicants), this scientific finding is further disclosed in US Patent No. 6291158 (**marked Exhibit A**), which generally teaches the generation of single domain (V_H or V_L) antibodies, and Riechmann et al., Journal of Immunological methods 231 (1999) 25-38 (**marked Exhibit B),** which demonstrates that isolated VH domains, can and do bind antigen with high affinity. Photocopies of **Exhibit A** and **Exhibit B** are provided herewith for the convenience of the Examiner.

At the time of Applicants' invention, it was known in the art that isolated V_H and V_L domains are capable of binding antigen. It was also known in the art that V_H and V_L domains contain fewer than six CDRs. Therefore, at the time of Applicants' invention, it was known to one skilled in the art that an antibody fragment with fewer that six CDR is capable of binding antigen with high specificity and affinity. This is in contradiction to the Examiner's assertion that all six CDRs are required for binding an antigen. Given that antibody fragments with fewer than six CDRs (*i.e* V_H and V_L domains), and the

method of making the same, was known to one skilled in the art at the time of Applicants' invention, Applicants' specification need not teach that which is known in the art.

Applicants, therefore, submit that antibodies and antigen binding portions thereof comprising less than six CDRs capable of binding an antigen with high affinities are known in the art, and Applicants were in possession of the claimed invention at the time the application was filed.

In an attempt to avoid any latent ambiguity concerning Applicants' claimed invention, Applicants have amended independent claim 22 by deleting the term 'CDR domain' from the phrase "variable region CDR domain." This amendment is supported throughout the specification as filed, and particularly at page11, lines 27-38 and page 12 lines 1-30. No new matter is added.

In the Office Action at page 3 and again at page 5, the Examiner restates the assertion that "Applicant appears to acknowledge that all six CDRs are required for the recited function in the response filed 3/28/2002." Applicants categorically deny this assertion for the record.

In the response filed 3/28/02, Applicants elect the CDR sequences as required by the restriction requirement. Applicants state that the heavy chain variable region may have 3 CDR sequences and the light chain variable region may have 3 CDR sequences. Applicants do not state that any antibody or antigen binding region cannot have fewer than 6 CDRs.

In view of the foregoing amendments and remarks, Applicants respectfully request withdrawal of the rejection of claims 22-38 under 35 USC §112 first paragraph.

In the Office Action, at page 4, paragraph 9, the Examiner has rejected claims 22-38 under 35 USC §112 first paragraph. The Examiner asserts that the specification, while enabling for antibodies and antigen binding fragments thereof in which 3 CDRs in the VH region and 3 CDRs in the VL region are defined, does not provide enablement for antibodies and antigen binding fragments thereof comprising less that three heavy chain and three light chain CDRs. The Examiner asserts that the Applicants have provided no working examples to show that fewer than all six CDR's provide the function of antigen binding. The Examiner also asserts that extensive and undue experimentation will be required to make antibodies with fewer than six CDRs capable of binding IL-18. Applicants respectfully disagree.

Applicants have previously stated USPTO examination guidelines that an applicant need not disclose, and preferably omits, that which is known in the art (see above).

It is well established that "when considering the factors relating to a determination of non-enablement, if all the other factors point toward enablement, then the absence of working examples will not by itself render the invention non-enabled." [MPEP § 2164.02].

The Examiner, while acknowledging the enabling disclosure of Ward et al., asserts that the art as a whole does not support the approach of Ward et al. Applicants respectfully disagree, and, have provided herein references establishing the fact that not all six CDRs of an antigen binding site are

necessary or utilized for binding a specific antigen, and that functional antibody fragments comprising fewer than all six CDRs is well known by practitioners skilled in the art. (See Applicants' remarks above). Once again, because it is known to persons skilled in the art that fewer than six CDRs are required for binding antigen, Applicants need not reiterate that which is known to those skilled in the art.

As stated *supra*, the fact that not all of the CDRs of the antigen binding site may be necessary (or even utilized) in binding a specific antigen, and that functional antibody fragments comprising fewer than all six CDRs is well known in the art, Applicants' specification as filed is fully enabled concerning such human antibodies and antigen binding portions thereof, which are capable of binding human IL-18. The absence of a working example for an antibody or antigen binding portion thereof comprising fewer than six CDRs and capable of binding IL-18 does not render Applicants invention non-enabled.

In addition to their enabling disclosure, Applicants have provided several references, available to one skilled in the art prior to the filing of Applicant's invention, which disclose antibodies with fewer than six CDRs capable of binding antigen with high affinities. For example, Ward *et al.*, (1989) *Nature* 341:544-546 (previously cited by Applicants), US Patent No. 6291158 (**marked Exhibit A**), and Riechmann et al., Journal of Immunological methods 231 (1999) 25-38(**marked Exhibit B**). Given Applicants' disclosure in concert with the references of what is known in the art, the skilled artisan would readily be able to generate antibodies with fewer than six CDRs to IL-18 with fairly routine and standard experimentation. Accordingly, Applicants respectfully submit that any experimentation that may be required to make and use the claimed invention, *i.e.*, antibodies with fewer than six CDRs to IL-18, constitutes routine, not undue, experimentation. The specification, therefore properly enables Applicants' claimed invention.

Applicants have amended independent claim 22 to recite more precisely Applicants invention, by deleting the term 'CDR domain' from the phrase "variable region CDR domain" such that it now reads "variable region". Independent claims 29, 30, 31, 36, 37 and 38 and dependent claims 31-35 are directed to an isolated antibody or antigen binding portion thereof with variable regions comprising certain amino acid sequences

In view of the foregoing amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 22-38 under 35 U.S.C. §112, first paragraph.

Claim rejections under 35 USC §103(a)

In the Office Action, at page 7, paragraph 14, claims 4-12, 14-24 and 44-46 are rejected under 35 USC §103(a) as being unpatentable over Kucherlapati et al., (US Patent No. 6,075,181) and Dinarello et al., (J. Leukoc. Biol. 1998; 63:658-664). The Examiner asserts the ordinary artisan at the time the

invention was made would have been motivated to combine the two cited references and produce fully human monoclonal antibodies that could bind and neutralize IL-18. Applicants respectfully disagree traverse the rejection.

BASIC REQUIREMENTS OF A *PRIMA FACIE* CASE OF OBVIOUSNESS

> To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all claim limitations.
>
> MPEP §2143

It is also well established that impermissible hindsight must be avoided (MPEP §2142).

Applicant's invention is directed to human antibodies or antigen binding portions thereof capable of binding human IL-18.

Kucherlapati et al. disclose a method of generating fully human antibodies to antigens. The Examiner acknowledges that Kucherlapati et al. do not teach human antibodies to human IL-18.

Dinarello et al. disclose recombinant human IL-18. Dinarello et al. disclose a clinical need for inhibiting IL-18 activity, potentially via an anti-IL-18 antibody. Dinarello et al. do not teach or suggest fully human antibodies to human IL-18.

There is no suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine the reference teachings (required as the first criterion to establish a *prima facie* case of obviousness) to produce a fully human antibody capable of neutralizing the activity of IL-18. Neither Kucherlapati nor Dinarello et al., either singularly or in combination, teach or suggest making human antibodies to human IL-18. The Examiner asserts that it would be obvious to one of ordinary skill in the art at the time the invention was made to make human antibodies to human IL-18. Applicants disagree. The very fact that, in view of the clinical importance of IL-18, neither reference teach or suggest a fully human anti-IL-18 monoclonal antibody is indicative (by its absence) that such an approach was unobvious to the authors (much less a person of ordinary skill in the art).

Applicants assert the Examiner has employed hindsight reconstruction, using Applicants' disclosure as a template, to improperly assert that it would be obvious to one skilled in the art to produce human antibodies capable of neutralizing activity of IL-18. Hindsight reconstruction of a claimed invention, absent a teaching or suggestion in the art is impermissible (MPEP §2142).

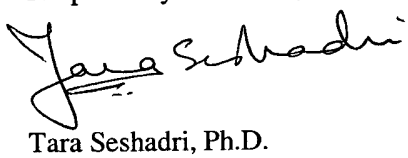
In addition, Applicants further assert there is no reasonable expectation of success, as required by the second criterion to establish a *prima facie* case of obviousness. Applicants teach how to make and use human antibodies to human IL-18. Neither the teachings of the cited references, nor the assertions of the Examiner, render Applicants' claimed invention obvious in the absence of Applicants' enabling disclosure.

In view of Applicants' foregoing remarks, Applicants submit that the rejection of claims 4-12, 14-24 and 44-46 under 35 U.S.C. §103(a) as being obvious over Kucherlapati et al., (US Patent No. 6,075,181) in view of Dinarello et al., (J. Leukoc Biol. 1998; 63:658-664), is improper. Applicants, therefore, respectfully request withdrawal of the rejection of claims 4-12, 14-24 and 44-46 under 35 U.S.C. §103(a).

Conclusion

In view of the foregoing amendments and remarks, Applicants believe the rejections set forth in the Office Action dated 16 December 2002 have been avoided or overcome and consequently their application is in condition for allowance. Applicants, therefore, respectfully request reconsideration and removal of the rejections, and allowance of the pending claims as amended.

Respectfully submitted,



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APPENDIX A

CLAIM AMENDMENTS UNDER 37 CFR §1.121(c)(1)(ii):
VERSION WITH MARKINGS TO SHOW CHANGES MADE

22. (Amended) An isolated human antibody, or an antigen-binding portion thereof, comprising at least one variable region [~~CDR domain~~] capable of binding an epitope of human IL-18.

EXHIBIT B

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Single domain antibodies: comparison of camel VH and camelised human VH domains

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Abstract

The antigen binding sites of conventional antibodies are formed primarily by the hypervariable loops from both the heavy and the light chain variable domains. Functional antigen binding sites can however also be formed by heavy chain variable domains (VH) alone. In vivo, such binding sites have evolved in camels and camelids as part of antibodies, which consist only of two heavy chains and lack light chains. Analysis of the differences in amino acid sequence between the VHs of these camel heavy chain-only antibodies and VH domains from conventional human antibodies helped to design an altered human VH domain. This camelised VH proved, like the camel VH, to be a small, robust and efficient recognition unit formed by a single immunoglobulin (Ig) domain. Biochemical, structural and antigen binding characterisation properties of both camel VH domains and camelised human VH domains suggest that these can compete successfully with single chain variable domain (Fv) fragments from conventional antibodies in many applications. Of special importance in this respect is the use of such VH domains as enzyme inhibitors, for which they seem to be better suited than Fv fragments. This function appears to be closely related to their often very long third hypervariable loop, which is central for antigen recognition in their binding sites.

Keywords: Antibody engineering; Immunoglobulin; VH; Camel; Single domain; Heavy chain

1. Introduction

Antibodies have long been considered as a powerful tool to recognise and target almost any molecule with a high degree of specificity and affinity. For such purposes, natural antibodies can be obtained as mixtures in the form of antisera from immunised animals (Cohn et al., 1949) or as monoclonal antibodies from hybridomas (Köhler and Milstein, 1975). More recently, recombinant DNA technology has allowed the cloning and genetic manipulation of antibody genes, which can then be expressed as recombinant antibodies in eukaryotic cells (Boulianne

Abbreviations: CDR, complementarity determining region; CH, heavy chain constant domain; CL, light chain constant domain; ELISA, enzyme-linked immunoadsorbent assay; Fab, antigen binding fragment; FR, framework; Fv, variable domain fragment; g3p, gene 3 protein; H1 to H3, heavy chain hypervariable regions 1 to 3; Ig, immunoglobulin; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; scFv, single chain Fv; T_m, melting point; VH, heavy chain variable domain; VL, light chain variable domain

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PII: S0022-1759(99)00138-6

et al., 1984; Neuberger et al., 1985). To obtain good yields of active antibody protein also from bacteria, it was necessary to change the antibody format from the full-length molecule, consisting of two multidomain heavy and light chains each, to smaller versions like the antigen binding fragment (Fab), or variable domain (Fv) fragment (Better et al., 1988; Skerra and Plückthun, 1988). These fragments all contain ordinary antigen binding sites formed by a single pair of N-terminal variable domains from heavy and light chain, but they contain no (Fv) or only one constant domain (Fab) per chain. Apart from enabling better expression in bacteria, these smaller formats have other advantages as they, for example, result in a better distribution and faster clearance than larger antibody molecules when used in vivo (Yokota et al., 1990). Their smaller size makes them also more suitable for structural studies like nuclear magnetic resonance (NMR) spectroscopy (McManus and Riechmann, 1991; Riechmann et al., 1991).

It was therefore attempted to create even smaller fragments of antibodies with adequate antigen binding activities. To decrease size significantly below that of Fv fragments, such minimal recognition units have to be based on single domains. And, indeed, already very early experiments indicated that antibody heavy chains can occasionally bind antigens in the absence of their light chain partner (Utsumi and Karush, 1964). These results were corroborated, when single heavy chain variable (VH) domains were isolated from bacterial expression libraries of heavy chain variable regions from immunised mice (Ward et al., 1989).

2. Camel heavy chain antibodies

While these results suggested that it might well be possible to generate single domain antibodies in vitro, it was then discovered that heavy chain-only antibodies had also evolved in vivo. In camels and other camelid species, a significant proportion of the natural antibody repertoire were found to consist of antibodies lacking a light chain partner (Hamers-Casterman et al., 1993). These antibodies have a molecular weight of ~95 kDa instead of the ~160 kDa for conventional antibodies (Ungar-Waron et al., 1987). At least, two different fractions of heavy chain antibodies could be separated from the conventional heterotetrameric antibodies by differential adsorption on proteins A and G. Later on, the sequence of cDNA clones of spleen or blood lymphocytes revealed the presence of three or four different heavy chain antibody isotypes in dromedary and llama, respectively (Vu et al., 1997). These cDNA sequences lack the exon coding for the first constant domain. The heavy chain polypeptide of heavy chain antibodies is therefore composed of the variable domain, immediately followed by the hinge, CH2 and CH3 domains (Fig. 1). The absence of the CH1 domain explains the absence of the light chain in the heavy chain-only antibodies, as this domain is the anchoring place for the constant domain of the light chain (Padlan, 1994). Besides the presence of immunoglobulin (Ig) heavy chains without CH1, the analysis of the cDNA clones revealed that sera of camelids contain also two different γ-isotypes with a CH1 domain (Vu et al., 1997). Evidently, these latter encode the heavy chains of the conventional antibodies within the Camelidae.

A pathological disorder in humans or mice, known as heavy chain disease, occurring in humans or mice is characterised by the presence of heavy chain antibodies in their sera (Seligmann et al., 1979). These truncated antibodies result from a somatic event that removes various parts of the VH and CH1 region from the expressed Ig gene. The mouse or human heavy chain antibodies however are not func-

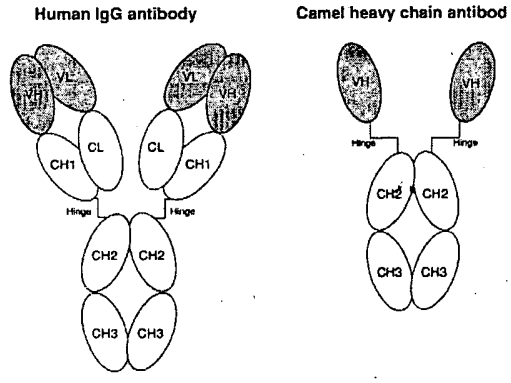

Fig. 1. Schematic picture of a conventional antibody (human γ-isotype) and a camel heavy chain antibody. The antigen binding site forming variable domains, which form either an Fv fragment in case of the conventional antibodies or a single domain antibody in case of the camel heavy chain antibodies, are shaded.

tional in antigen binding. In sharp contrast, the blood sampling of infected or immunised dromedaries showed that the immune response contained a diverse repertoire of heavy chain-only antibodies, which were functional in antigen binding (Hamers-Casterman et al., 1993).

3. Camelising human VH domains

Sequence analysis suggested that the camelid VH domains may contain a significantly altered surface in that region, which in VHs from conventional antibodies forms the VH/light chain variable domain (VL) interface (Chothia et al., 1985; Muyldermans et al., 1994). The antibody VH gene family, which is overall most homologous to VHs from camel heavy chain-only antibodies, is the human VH3 family (Muyldermans et al., 1994; Nguyen et al., 1998). Their framework (FR) sequences are very similar except for three residues in FR2 (Fig. 2), which are highly conserved in VH domains from most conventional antibodies. These residues (G44, L45, W47 in the human VH3; E44, R45, G47 in most camel VHs) are located in the VH/VL interface and the side chains of residues 45 and 47 point towards the VL in structures of conventional antibodies (Chothia et al., 1985).

To analyse the effect of these mutations, a human VH3 was expressed as an isolated domain in *Escherichia coli* and the three mutations were introduced in its former VL interface (Davies and Riechmann, 1994). The original human VH and two camelised mutants (G44E, L45R, W47I or W47G) were all found to be monomeric at low protein concentrations. However, the original human VH, in the absence of a VL domain, started to aggregate at higher protein concentrations (1 mg/ml and above) causing severe line broadening in NMR analysis (Davies and Riechmann, 1994). Aggregation was significantly reduced for the camelised VH domains. Thus, the camelised human VH domains had a much improved linewidth (transverse proton relaxation time T_2 was increased from 14.5 up to 29 ms) in NMR

```
FR1          1       5        10        15        20        25        30
Human VH3    E V Q L V E S G G G L V Q P G G S L R L S C A A S G F T F S
             . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Camel VH     E V Q L V E S G G G S V Q A G G S L R L S C A A S G Y T Y S

CDR1/FR2  31       35    36       40        45      49
Human VH3    @ @ @ @ @     W V R Q A P G K G L E W V S
                           . . . . . . . * * . * . .
Camel VH     @ @ @ @ G     W F R Q A P G K E R E G V S

CDR2      50  52 a53  55         60        65
Human VH3    @ @ @ @ @ @ @ @ @ Y A D S V K G
                               . . . . . . . .
Camel VH     @ @ @ @ @ @ @ @ @ Y A D S V K G

FR3       66       70        75        80  82 a b c83  85        90       94
Human VH3    R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A R
             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Camel VH     R F T I S Q D N A K N T V Y L Q M N S L K P E D T A I Y Y C A A

CDR3/FR4  95        100 102   103 105        110   113
Human VH3    @ @ @ @ @ @ @ @     W G Q G T L V T V S S
                                 . . . . . . . . . . .
Camel VH     @ @ @ @ @ @ @ @     W G Q G T Q V T V S S
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Fig. 2. Consensus VH sequences from camel heavy chain-only antibodies (Muyldermans et al., 1994) and the human VH subgroup 3 (Kabat et al., 1991). The most frequent amino acid at each position is given except for residues 31 to 35, 50 to 58 and 95 to 102 (@), which are hypervariable in both VH families. Residues 44, 45 and 47 (*) were altered to camelise a human VH3 domain (see text). Residues identical in the camel VH and human VH sequence are indicated by a dot. FR1 to FR4 denote framework regions, CDR1, CDR2 and CDR3 denote complementarity determining regions and contain the hypervariable regions.

experiments, which enabled their structural analysis in solution (Davies and Riechmann, 1994; Riechmann, 1996; Riechmann and Davies, 1995). A very limited number of mutations in the former VL interface had therefore made it possible to create a human single VH domains, which behaved well in solution.

Apart from enabling the NMR analysis of the VH domain, this improvement opened the possibility to create single domain recognition units based on human heavy chain variable domains. Such camelised human VH domains may turn out to be more suitable than camel VH domains for any future in vivo use. They further carry, in case of the human VH3 domain, the additional advantage of being recognised by the bacterial superantigen protein A, which facilitates highly specific and efficient purification. Protein A binds to a nonlinear epitope within the VH (Riechmann and Davies, 1995) and thereby will result in purification of correctly folded VH protein only.

4. Preparation of specific camel heavy chain antibodies

The immunisation of llamas and dromedaries following standard protocols (complete and incomplete freund adjuvant; three to four boosts with 50 μg to 1 mg immunogen per animal) generates specific heavy chain-only antibodies of good titres. Approximately 0.1 mg of polyclonal heavy chain-only antibodies with specificity for α-amylase could be trapped by batch adsorption from 1 ml serum of an immunised dromedary (Lauwereys et al., 1998).

The polyclonal heavy chain-only antibodies of the IgG3-type purified on proteins A and G can also be used as a source to isolate variable domains. These variable domains are then obtained after a limited proteolytic digestion of IgG3 with endo-Glu V8 protease. This enzyme cleaves the short hinge region between the VH and CH2. Protein A chromatography retains the Fc containing fragments and a subfraction of the camel VHs. The flow-through contains the majority of the VH domains, of which measurable amounts could recognise the antigen (Lauwereys et al., 1998).

To obtain recombinant forms of camel VH domains, the repertoire of heavy chain-only antibody variable domains from an immunised camel are cloned in bacteria (Ghahroudi et al., 1997). Expression libraries of such VH repertoires can be screened for the presence of antigen specific binders. To avoid the contamination with VH genes originating from conventional antibodies a two-step polymerase chain reaction (PCR) was proposed (Ghahroudi et al., 1997). In the first PCR on cDNA template of blood lymphocytes a set of primers, which anneals at the first codons of the VH and within the CH2 region, were used to amplify all γ-isotypes. The PCR products originating from the heavy chain-only antibodies could be selectively eluted after gel electrophoresis since the fragment of the heavy chain-only antibody γ-isotypes are ~350 nucleotides shorter due to the absence of the CH1 exon. The entire VH region is then reamplified with nested primers annealing at the codons of FR1 and FR4 of the VH, respectively. The resulting PCR product is finally ligated in a phage display vector (see below) adapted to clone a VH region only. A VH library of 10^6–10^7 individual clones, from which typically several antigen binders were selected, can be obtained from as little as 5–10 ml blood. The whole procedure from the first immunisation to the identification of binders can be performed in less than 3 months. Following this procedure, more than 20 binders against a variety of proteinaceous antigens were isolated (Table 1). These VHs bound their antigen specifically with dissociation constants from 100 nM into the subnanomolar range (Muyldermans and Lauwereys, 1999). This affinity is similar to that measured for conventional antibodies from a secondary immune response (Foote and Milstein, 1991).

Although phage display or expression libraries for Fv or Fab fragments of conventional antibodies can also be prepared from blood of immunised or infected mice and humans (Persson et al., 1991; Winter et al., 1994), the single domain V_HH libraries of immunised camels have several advantages. First, all the variable domains of camelid heavy chain antibodies belong to one single family (VH3). Such a VH library can therefore be generated with a single set of PCR primers. Secondly, the entire paratope is located on a single domain present in a single exon that is cloned as one entity. In contrast, the VH genes of mouse and human antibodies belong to several families so that multiple PCRs with different sets of primers are necessary to clone the complete

Table 1
Affinities of camel VH domains and camelised human VH domains
Data for the camel VH are from Ghahroudi et al. (1997), Lauwereys et al. (1998) and K. Conrath (personal communication), data for the camelised human VHs are from Davies and Riechmann (1996b). The antigen used were α-amylase, carbonic anhydrase, β-lactamase, 4-glycyl-2-phenyloxazol-5-one (OxGly), 3-iodo-4-hydroxy-5-nitrophenyl-acetyl caproic acid (NIP-CAP), the HIV transcription factor rev and hen egg lysozyme. The length of the H3 region refers to the number of residues between 95 and 102 inclusive using the Kabat nomenclature (Kabat et al., 1991).

	Ligand	K_d (nM)	H3-length
Camel VH			
cAb-AMD7	α-amylase	15	16
cAb-AMD9	α-amylase	3.5	14
cAb-AMB10	α-amylase	24	14
cAB-CA04	carbonic anhydrase	29	18
cAb-CA06	carbonic anhydrase	20	13
cAb-Lys2	lysozyme	2	19
cAb-Lys3	lysozyme	65	24
cAb-βla01	β-lactamase	<1	21
cAb-βla02	β-lactamase	1.5	8
cAb-βla03	β-lactamase	10	17
Camelised VH			
VH-Ox21	OxGly	146	15
VH-OS1	OxGly	25	15
VH-Ox21.2.4	OxGly	47	15
VH-Ox62	OxGly	267	15
VH-N3c1	NIP-CAP	292	15
VH-N3c1.2.2	NIP-CAP	31	15
VH-REVg1	rev	220	11
VH-REVrs1	rev	401	11
VH-LS2	lysozyme	3100	10
VH-LS2.5.1	lysozyme	1600	10

repertoire. In addition, the Fv has to be reconstituted from the combination of a VH and a VL domain. These must be amplified as separate gene fragments, which are randomly combined afterwards. Therefore, original pairs of VH and VL domains with antigen binding activity that were matured during the immune response as one entity can only be recovered by random combination requiring Fv libraries of relatively large size, which at times approaches the limitations of bacterial transformation efficiency.

5. Preparation of specific camelised VH domains

While camel heavy chain-only antibodies of a desired antigen specificity can be rescued from natural sources, camelised VH domains are synthetic and must therefore be generated in vitro. For this, the human VH3 domain with the camelised VL interface (i.e., with mutated residues 44, 45 and 47) was used as a building block for the creation of designed libraries. VH repertoires can most simply be created through the introduction of randomised regions into the VH gene to vary the hypervariable loops both in length and amino acid residue nature. The three hypervariable loops of VH and VL form the antigen binding site in ordinary antibodies (Chothia et al., 1989).

To facilitate selection of a repertoire of camelised VH domains, these were displayed, like other antibody fragments before, on filamentous phage by fusion to the N-terminus of the minor phage coat gene 3 protein (g3p) on the gene level (Davies and Riechmann, 1995a). Each phage particle displays its individual VH, which is encoded in the encapsulated phage genome. VHs can therefore be selected, for several rounds if needed, through panning of phage on immobilised antigen. Bound phage is rescued and regrown through infection of bacteria. Selected VHs can be analysed (and indeed be used for detection or targeting) for their antigen specificity and affinity as phage displayed VH or as soluble VH. Soluble VH can be produced after either subcloning into a soluble expression vector or through the use of bacterial suppresser strains and appropriate stop codons between the VH fusion and the phage g3p (Hoogenboom et al., 1991).

Repertoires of camelised VH domains were initially created by randomisation of residues within the third hypervariable loop H3, which at the same time was varied in length. In VH domains from conventional antibodies and indeed camel heavy chain-only antibodies, this loop contains among the three VH hypervariable loops the highest diversity in length and amino acid nature (Kabat et al., 1991; Wu et al., 1993). From such a repertoire of 2×10^8 clones, camelised VH domains specific for hapten, peptide and protein antigens were selected (Davies and Riechmann, 1995a, 1996a; Martin et al., 1997). The dissociation constants for these VH domains in their soluble form and their respective antigens were in the nanomolar to micromolar range (Table 1). The affinities could be improved up to tenfold by phage selection of VHs containing secondary mutations

within the other two hypervariable loops (Davies and Riechmann, 1996b).

These results show that camelised VH domains present a useful building block for the preparation of Ig-based recognition units of minimal size. Selected VH domains proved to be highly specific and of reasonable affinity, which can be increased through the preparation of larger repertoires or secondary mutations.

The same experiments, however, also made obvious that some properties of camelised VH domains can be improved upon. These are related to the former VL interface of the VH. The three camelising mutations (G44E, L45R and W47G), which increased the solubility of the single human VH domain most and lead to the least nonspecific binding of phage displayed VH domains, compromised the stability and most significantly the expression yield of active protein for the resulting soluble VH (Davies and Riechmann, 1994, 1995a). Camelised VH domains, which had been selected with a glycine at position 47, were therefore expressed as G47I mutants in their soluble version. This had in most cases minor or no effects on antigen affinity. The G47I mutation increased in all cases stability and purification yields of active VH protein significantly. Unfortunately, constitutive use of isoleucine at position 47 of the VH for phage display lead to an unacceptably high number of nonspecifically binding clones in the repertoire, which compromised selection (Davies and Riechmann, 1995a).

Further mutations in the camelised, human VH domain appear to be necessary to create a prototype FR, which exhibits low nonspecific binding and high expression yields at the same time. Thus, it was indeed possible to improve protein stability of camelised VH domains significantly through the introduction of a new intradomain disulphide bridge between cysteines at positions 33 (in FR2) and 100b (in H3), which could even be constitutively introduced in repertoires of camelised VH domains (Davies and Riechmann, 1996a).

Another problem concerns the multimerisation state of the selected VH domains. While most selected camelised VH domains were monomeric, dimeric domains also were occasionally selected (Martin et al., 1997). This obviously depends on the nature of the H3 loop as this is the only portion of the camelised VHs, which is different among the VH domains in the original repertoire.

Concerning the generation of diversity within repertoires, increased variation both in length and sequence of the H3 loop in camelised VH domains is probably the most effective and simplest method. Sequences from antigen specific, natural camel heavy chain antibodies after immunisation suggest that the H3 loop has an even more central role for antigen binding in heavy chain-only antibodies than it has in conventional antibodies (Muyldermans et al., 1994). Repertoires based on completely randomised H3 loops can be further diversified through a limited number of mutations in the other two hypervariable loops.

6. Expression of recombinant heavy chain-only antibodies and single VH domains

6.1. Phage antibody fragments

For the phage display of antibodies, these are typically fused as Fab or single chain Fv (scFv) fragments to the phage g3p protein. Once a specific antibody clone has been selected, this can be used for many applications directly in this phage format (Nissim et al., 1994). For example, selected phages can be most easily screened from supernatants of infected bacteria in enzyme-linked immunoadsorbent assay (ELISA) for antigen binding, where they are detected with an anti-phage antibody (e.g., anti-M13 monoclonal antibody; Pharmacia). This essay is highly sensitive as the phage coat contains about 2700 copies of the major phage coat protein gene 8 protein (Model and Russel, 1988), which forms the epitope for the anti-phage antibody. When a pure phage system (rather than a phagemid in combination with a helper phage) is used to provide the g3p-fusion, the phage contains up to five copies of the antibody fragment resulting in avidity effects, which will allow the detection of even very weak binders.

Concerning the use of phage displayed antibody fragment, there is no difference between ordinary heavy and light chain antibody fragments and single chain polypeptide antibodies like the camel heavy chain-only antibodies or camelised human VH do-

mains. VHs from camel heavy chain antibodies and the camelised human VH domains have both been successfully fused to g3p (Davies and Riechmann, 1995a; Ghahroudi et al., 1997) and were suitable for detection of their antigens in ELISA. Their use in other application, like histological staining or cell sorting, has not been tried, but there is no reason why they should perform any worse or better than ordinary antibodies.

6.2. Soluble antibody fragments of camel heavy chain-only antibodies

The genes for camel VH domains can be inserted by cassette mutagenesis into suitable expression vectors to produce larger multidomain or multifunctional proteins. For example, an intact heavy chain-only antibody was readily generated by cloning a particular camel VH in front of the hinge and effector function domains of human IgG1. This construct can be expressed in bacteria, but expression in mammalian cell lines will add the proper glycosylation at the CH2 domain. Such constructs in a pcDNA3 vector (Invitrogen) produced after transient expression in COS cells some 5 mg/l medium (K.B. Vu, personal communication). These chimeric heavy chain antibodies were fully active in antigen binding.

Multimerised forms of camel VHs linked on a single gene through a peptide were also obtained from bacteria yielding multivalent recognition units (K. Conrath, personal communication). Two camel VHs directed against different antigens should form bispecific recognition units when linked on the gene level. Equivalent constructs have been made using conventional scFv as molecular building blocks (Neri et al., 1995). However, the single domain nature of the camel VH will most likely yield higher functional expression levels.

We are convinced that camel VH will be most useful in diagnostic applications where stable and, small reporter molecules are required. Camel VHs can routinely be expressed with a His tail in the bacterial periplasm. The average purification yield obtained after purification by immobilised metal affinity chromatography and gel filtration is between 0.5 and 5 mg/l culture. These recombinant camel VHs do not dimerise and can be concentrated to 10 mg/ml without aggregation. The VHs are stable, they resist incubations at 37°C for 1 week and T_m values between 60°C and 72°C were measured (Ghahroudi et al., 1997).

6.3. Soluble camelised human VH domains

Camelised VH domains, which were selected from phage display libraries, have been expressed as soluble VH domains after subcloning into *E. coli* expression vectors directing folded protein to the periplasm through fusion to a suitable leader peptide (Davies and Riechmann, 1995a). Camelised VHs containing a glycine at position 47, which was most successfully used for selection after phage display, yielded up to 1 mg functional protein from 1-l bacterial culture grown in shaker flasks using protein A sepharose for purification from both supernatants or periplasmic preparations. The same VH domains, expressed with an isoleucine at position 47, yielded up to 10 mg active protein from 1 l of culture. The same was usually the case even when the VHs contained two additional cysteines (Davies and Riechmann, 1996a). ScFvs rescued from phage display libraries or indeed engineered from monoclonal antibodies have often similar purification yields (Skerra, 1993), which however can be highly variable depending on the particular scFv used. This is due to the wider structural variety of scFvs, while most camelised VHs, which are identical except for their H3 loop, give purification yields in the discussed range. In addition, expression of both camel VH domains and camelised human VH domains is not compromised by the presence of a linker between VH and VL as in a scFv. Such linkers can interfere with folding and can be susceptible to proteolysis (Whitlow et al., 1993).

Concerning the stability of the camelised VH domains, these are usually considerably higher than those of scFv or ordinary Fv fragments, which can fall more easily apart due to their two-domain architecture. The camelised VH domains containing a glycine at position 47 have melting points of about 60°C, while those with an isoleucine at position 47 have a T_m of about 70°C (Davies and Riechmann, 1995b, 1996a). Other mutations, like the change of valine 37 to phenylalanine and the introduction a second intradomain disulphide bond between newly

introduced cysteines at positions 33 and 100b, which were also adapted from natural camel VH domains, increased the thermostability of the VH domains to almost 80°C (Davies and Riechmann, 1996a). Again, as with purification yields, stabilities of scFvs are much more variable due to their more diverse nature of underlying VH and VL genes in addition to the variable lability of the VH/VL interaction.

Other features of camelised VH domains are less straightforward to compare with those of other formats of antibody fragments. Thus, camelised VH domains with long third hypervariable loops were occasionally cleaved by bacterial proteases. Although this may also be occasionally observed in scFvs, it will be less frequent in the case of conventional antibody combining sites as these contain usually shorter H3 loops.

Another observation is the formation of dimeric, camelised VH domains (Martin et al., 1997). Dimerisation is also not unknown for scFvs. In the case of scFvs, it was possible to turn that into a feature rather than a problem. Through the use of short VH/VL linkers, dimerisation (and even trimerisation) can even be enforced and can lead to so-called diabodies with two identical binding sites, or even different specificities when VH and VL domains from two different scFvs are linked on the same gene (Holliger et al., 1993). In the case of single VH domains, dimerisation through covalent linkage of two different VHs should also lead to avidity effects or to the creation of dual specificity. However, such modifications will compromise the two most important advantages of VH domains, which are small size and high stability.

As far as aggregation of camelised VH domains compared to other antibody fragments is concerned, this will very much depend on the nature of the particular fragment. Most camelised VH domains or Fv and Fab fragments do not aggregate, however, occasionally fragments are prone to aggregation depending on particular sequences within the proteins.

7. Structures of camel VH domains

The crystal structures of one llama and two dromedary VH domains are available in the protein data bank. The llama VH with specificity for human chorionic gonadotropin was solved to 1.8 Å resolution (pdb-file 1HCV) (Spinelli et al., 1996). The dromedary VHs, cAb-Lys3 (pdb-file 1MEL) cAb-RN05 (pdb-file 1BZQ) and cAb-RN05 (pdb-file 1BZQ), were crystallised with their respective antigens, hen egg-white lysozyme and bovine RNase A (Desmyter et al., 1996; Decanniere et al., 1999; Fig. 3). In all three cases, the Ig fold of the VH is well preserved. Two β-sheets (one with four and one with five β-strands) are packed against each other and stabilised by a conserved intradomain disulphide bond between C22 and C92. The rms deviation between the core of any two camel or llama VH and human VH (of family 3) is between 0.27 and 0.69 Å (Decanniere et al., 1999).

The side of the camel VH domain corresponding to the VL interacting face of the normal VH in an Fv has a quite different architecture. Compared to the human VH, four amino acid substitutions (V37F, G44E, L45R and W47G or W47S) are located in this region. The substitutions at positions 44, 45 and 47 were those, which were used to camelise the human VH and rendered the isolated domain more soluble (Davies and Riechmann, 1994). The nonpolar to polar amino acid substitutions (G44E and L45R) increase the hydrophilicity of the surface. The substitutions at positions 37 and 47 cause a net shift of the bulky hydrophobic groups. In case of the two camel VHs with a known structure, the H3 loop folds over these residues and makes them solvent inaccessible (Decanniere et al., 1999).

From a survey of all human and mouse VH antigen binding loop structures, it became apparent that only a restricted number of possible conformations were encountered (Chothia and Lesk, 1987; Chothia et al., 1992; Al-Lazikani et al., 1997). Three and four different conformations are described for the first and second antigen binding loop, respectively. These so-called canonical structures are determined by the length of the loop and the presence of particular residues at key positions. The H3 loop is extremely variable in length and sequence (Wu et al., 1993). Consequently, the prediction of the loop architecture remains more speculative despite recent progress (Martin and Thornton, 1996; Shirai et al., 1996; Morea et al., 1998). Surprisingly, the antigen binding loop structures of camel VHs deviate from the present canonical loop definitions of human and

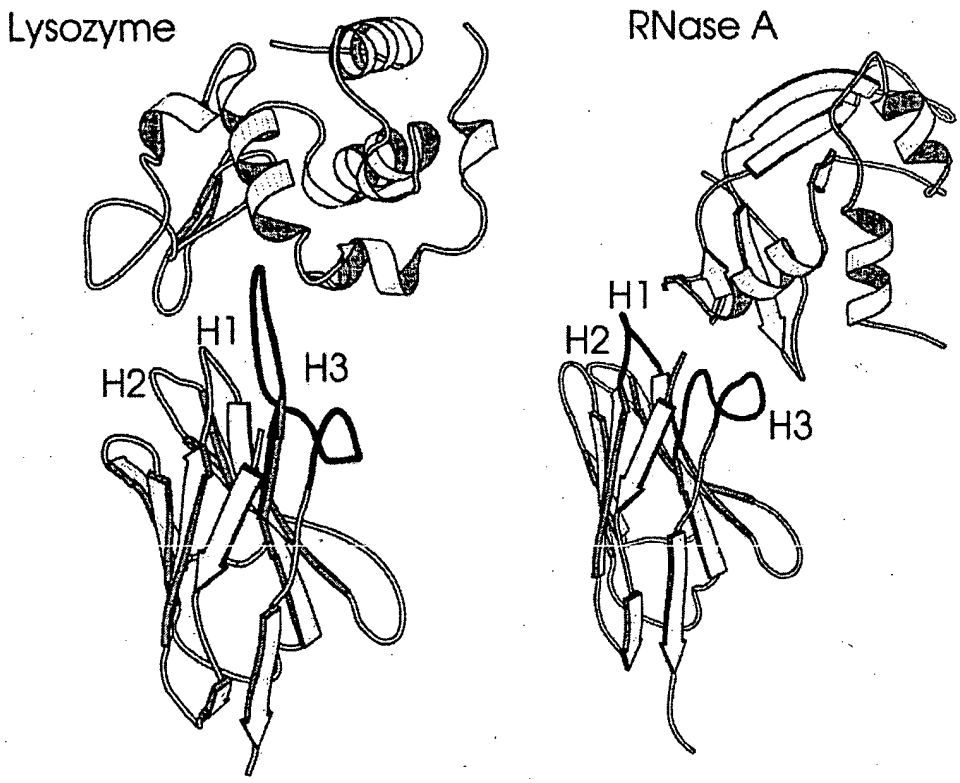

Fig. 3. Crystal structures of the complexes between two camel VH domains and their antigens. The complex cAb-Lys3::lysozyme (left) illustrates how the long H3 loop (black) protrudes from the remaining paratope and inserts into the catalytic site of lysozyme. In the cAb-RN05::RNase A model (right) only two VH loops, H1 and H3 (black) are involved in antigen binding. The H3 loop of the cAb-RN05 antibody (12 amino acids) is only half of that of cAb-Lys3. The H3 loop of cAb-RN05 does not protrude from the remaining paratope but folds over the former VL interface.

mouse VHs. This deviation could not be predicted, because the loop length and the residues at the key positions are very similar between camel VH and human VH. Consequently, the prediction of the camel VH hypervariable loop structures by the current canonical loop structure algorithms is not reliable. The additional canonical loop structures in camel VHs make the structural repertoire of their paratope larger than that of VH domains in Fv fragments from conventional antibodies.

Moreover, the hypervariable region around the first antigen binding loop in VHs of camel heavy chain-only antibodies is enlarged to cover residues 26 to 35 (Vu et al., 1997). In human VHs, the first hypervariable region only comprises positions 31 to 35 (Kabat et al., 1991). This indicates that residues 26 to 30 in camel VHs may more frequently participate in antigen binding. Indeed residue 29 (I29) of cAb-Lys3 interacts directly with the antigen lysozyme (Desmyter et al., 1996) and the hydroxyl group of Y27 of cAb-RN05 is hydrogen bonded with its RNase A antigen (Decanniere et al., 1999). The first amino acid of conventional VHs, which usually interacts with antigen, is located at position 30 (Tomlinson et al., 1996). The extension of the first hypervariable region in camel VHs and a concomitant enlarged antigen binding surface compared to that of a VH in a conventional antibody appears to compensate in part for the absence of a VL domain.

However, the antigen binding surface of cAb-RN05 remains small (570 $Å^2$) compared to Fv paratopes interacting with proteins (613–841 $Å^2$) due partly to a lack of participation of the H2 loop in antigen binding (Fig. 3). A large number of main

chain hydrogen bonds and van der Waals contacts leads nevertheless to an dissociation constant with antigen of 30 nM.

The cAb-Lys3 has a very long H3 loop of 24 residues, of which the first 10 protrude from the antigen binding site (Fig. 3). As a consequence, the actual antigen binding surface (847 Å^2) becomes even larger than that of most conventional Fvs (613–841 Å^2). The protruding H3 loop is constraint by a disulphide bond towards the H1 loop and by the parallel stacking of two tyrosines in the interior of loop H3 (Desmyter et al., 1996).

No antigen binding data are available for the llama VH. However, its short H3 loop (Spinelli et al., 1996) suggests that the actual antigen binding surface will be correspondingly small.

8. Structure of a camelised VH domain

The structure of one camelised human VH domain was solved in solution by NMR spectroscopy (pdb-file 1VHP). This VH contains three camelising mutations (G44E, L45R and W47I) in its former VL interface. The overall β-sheet structure is very similar to that of a noncamelised, VL-associated human VH3 domain (Pot; Fan et al., 1992) with a C^{α} rms of 2 Å for 82 (out of 113) aligned residues (Riechmann, 1996). The first two hypervariable loops of the camelised VH and the Pot-VH have the same length and adopt the same canonical structures. The H3 loop folds in the Pot-VH towards the VL and participates in VH/VL interactions. The (by four residues shorter) H3 loop in the camelised VH in contrast is orientated more towards the H1 and H2 loops. This is also in conflict with the camelid VH domains where the H3 loop, or part of the H3 loop, covers the side of the VH, which would form the VL interface in conventional antibodies. In the camelid VHs, the H3 loop covers hydrophobic regions of the VH, which would otherwise be exposed to the solvent. The camelising mutations reduce the hydrophobic character of the protein surface both in the camel and the camelised VHs, as three hydrophobic residues (44, 45 and 47) are replaced by more hydrophilic amino acids. Hydrophobicity of the former VL interface in the camelised VH was further reduced by the reorientation (compared to a VL-associated VH) of the side chains of the nonmutated residues 37, 38 and 103. The hydrophobic side chains of residues V37 and W103 are completely or partly buried in the camelised VH, while they point towards to the VL in a VL-associated VH. The opposite is the case for the hydrophilic R38 side chain. These changes were not found in the camel VH structures and indicate a principle structural difference between camel and camelised VH domains. Whether this will also be true for camelised VHs with extremely long H3 loops as in natural camel VHs remains to be seen. Folding back of a long H3 loop onto the former VL interface in camelised VH domains might well influence its structure.

No direct structural details are available for antigen binding by camelised VH domains, but affinity improvement due to changes in the H1 and H2 loops suggest that these may also be involved in antigen binding (Davies and Riechmann, 1996b). The H3 loop will almost certainly play a central role in their binding site as it formed the source of structural variety in the underlying repertoire, from which the VH domains were selected.

9. Special features of single domain antibodies

The observation of a convex paratope architecture in the camel VH cAb-Lys3 (Desmyter et al., 1996) was most remarkable since it has never been observed in Fvs (Padlan, 1996). Normally, the antigen binding loops of an Fv form a cavity, a groove or a flat surface (Webster et al., 1994). These Fv topographies are correlated with the size and type of antigen. Haptens tend to be bound into cavities of the paratope, peptides bind into a groove, and larger antigens such as proteins are bound by antibodies with flat paratopes, eventually undulated by some side chains to improve the complementarity of the surfaces (Padlan, 1996). Large protruding loops of 10 amino acids or more seem to be unique for the antigen binding sites of heavy chain-only antibodies. This feature gives camel VHs a special niche for antigen recognition, as their long third hypervariable loop can insert into cavities of antigen surfaces. This is especially important, as the catalytic site of en-

zymes is often located at the largest cavity on their protein surface (Laskowski et al., 1996). Such sites are usually not immunogenic for conventional antibodies (Novotny et al., 1986). Camel heavy chain-only antibodies however are able to recognise such epitopes.

Indeed, in the structure of camel VH cAb-Lys3, the 24 residue long H3 loop penetrates deeply into the active site of lysozyme (Transue et al., 1998) suggesting that camel heavy chain antibodies might be able to form specific enzyme inhibitors. Proof was given by the successful retrieval of specific inhibitors from the VH library of a camel immunised with α-amylase, lysozyme and carbonic anhydrase (Lauwereys et al., 1998). Similarly, from a library of camelised VHs with randomised third hypervariable loops (Davies and Riechmann, 1995a) an inhibitor for the hepatitis C virus NS3 protease could be selected in vitro (Martin et al., 1997).

Whether inhibition is indeed in all cases due to the insertion of long hypervariable loops into active site cavities of the enzymes will have to be confirmed by structural studies, and it cannot be excluded that inhibitory VHs might have additional strategies to inactivate enzymes.

The paratope of camel VHs comprises only three antigen binding loops, of which H3 provides most of the contacts. The lower complexity of their antigen binding site might therefore make single domain antibodies a suitable FR for the peptide scanning technique (Laune et al., 1997) to design smaller peptides or peptide analogues with enzyme inhibiting or receptor blocking capacity derived from the sequences of the H3 loops.

10. Conclusions

The most basic difference between camel VHs and camelised VHs is that camel VHs have naturally evolved and can be obtained by in vivo immunisation. While immunisation can be mimicked in vitro through selection of synthetic phage displayed VH domains, the architecture of the camel VHs is at present still superior to that of camelised VHs because of a more mature design as a result of their natural evolution. Camelised human VH domains may need additional modifications in their now exposed, former VL interface to tolerate the exposure to a hydrophilic surrounding while maintaining a high stability and good folding properties and providing a highly diverse antigen binding site. Once this disadvantage is overcome, camelised and camel VH domain should be equally well suited for the in vitro selection of antigen specific single domain antibodies. Concerning the in vivo selection of VH domains with good binding properties after immunisation of animals, camel heavy chain-only antibodies will obviously remain unchallenged. Advantages of camelised, human VHs compared to camel VH domains however include the presence of a Protein A binding in the case of domains based on the human VH3 gene family and the possibly more attractive use of camelised, human VHs for any therapeutic purpose in humans due to a probably lower immunogenicity.

Given a camel available for immunisation and an antigen ready for injection, it is relatively straightforward to generate a recombinant camel single domain antibody with good affinity and specificity. This may prove particularly valuable when specific enzyme inhibitors are needed, as these seem to be very rare among conventional antibodies but frequent among camel heavy chain antibodies. This property appears to be closely related to their often very long H3 loop. This niche for antigen binding together with the advantages in size and stability should lead to an important role for single VH domains for biotechnological applications in the future.

It seems therefore also desirable to establish in vitro systems for the generation of such single domain antibodies avoiding the need for animals. To prepare single domain antibodies based on natural camel VHs in vitro, a large bank of the VH germline segments, which are used in the camel heavy chain-only antibodies, must be prepared and for example completed with synthetic H3 loops and a suitable C-terminal FR. Their display on phage will allow in vitro selection of specific recombinant VH domains. Camelised, human VH domains present a viable alternative to camel VHs. These however may still require some improvements to efficiently compete with the more reliable biochemical properties of natural camel VH domains. Such a design may eventually lead to domains even smaller than full-

length VH domains, as already shown in the case of the VH-based β-domains (Pessi et al., 1993; Martin et al., 1994). Alternatively, it may also be possible to utilise light chain variable domains for this purpose, although these lack at least in nature the feature of a frequently very long hypervariable loop.

In any case, single VH domains have so far proven to perform no worse than Fv or Fab fragments from ordinary antibodies concerning antigen binding and purification yields. In addition, single VH domains are definitely more stable due to their simpler architecture. They are therefore likely to challenge conventional antibodies for many biotechnological applications, while in most therapeutic applications conventional antibodies will remain the reagent of choice for reasons of immunogenicity. Possible exceptions even in therapy are antibody-based enzyme inhibitors, as only camel heavy chain-only antibodies seem to perform this function in vivo, and anti-idiotypic vaccinations, as camel VHs should raise good responses in noncamelid species due to their noncommon structures of antigen binding loops.

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